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*DOI:*
10.1016/j.aca.2018.03.019

Analytica Chimica Acta xxx (2018) 1–15

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

# Comparison of modified Matyash method to conventional solvent systems for polar metabolite and lipid extractions

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## HIGHLIGHTS

- Bligh and Dyer, Matyash and a new 'modified Matyash' solvent systems were compared.
- Applied to three sample types and analysed by mass spectrometry-based metabolomics.
- Modified Matyash showed comparable or higher extraction yield than other methods.
- Reproducibility of modified Matyash method was also comparable or higher.

## ARTICLE INFO

*Article history:*
Received 31 October 2017
Received in revised form
13 March 2018
Accepted 18 March 2018
Available online xxx

*Key words:*
Metabolic phenotyping
Lipidomics
Sample extraction
Phase separation
Chloroform
MTBE

## ABSTRACT

In the last decade, metabolomics has experienced significant advances in the throughput and robustness of analytical methodologies. Yet the preparation of biofluids and low-mass tissue samples remains a laborious and potentially inconsistent manual process, and a significant bottleneck for high-throughput metabolomics. To address this, we have compared three different sample extraction solvent systems in three diverse sample types with the purpose of selecting an optimum protocol for subsequent automation of sample preparation. We have investigated and re-optimised the solvent ratios in the recently introduced methyl tert-butyl ether (MTBE)/methanol/water solvent system (here termed modified Matyash; 2.6/2.0/2.4, v/v/v) and compared it to the original Matyash method (10/3/2.5, v/v/v) and the conventional chloroform/methanol/water (stepwise Bligh and Dyer, 2.0/2.0/1.8, v/v/v) using two biofluids (human serum and urine) and one tissue (whole *Daphnia magna*). This is the first report of the use of the Matyash method for extracting metabolites from the US National Institutes of Health (NIH) model organism *D. magna*. Extracted samples were analysed by non-targeted direct infusion mass spectrometry metabolomics or LC-MS metabolomics. Overall, the modified Matyash method yielded a higher number of peaks and putatively annotated metabolites compared to the original Matyash method (1–29% more peaks and 1–30% more metabolites) and the Bligh and Dyer method (4–20% more peaks and 1–41% more metabolites). Additionally the modified Matyash method was superior when considering metabolite intensities. The reproducibility of the modified Matyash method was higher than other methods (in 10 out of 12 datasets, compared to the original Matyash method; and in 8 out of 12 datasets, compared to the Bligh and Dyer method), based upon the observation of a lower mRSD of peak intensities. In conclusion, the modified Matyash method tended to provide a higher yield and reproducibility for most sample types in this study compared to two widely used methods.



## 1. Introduction

Metabolomics has now matured into a routinely used technology for measuring the metabolic phenotypes of a wide array of sample types – including biofluids, cells and tissues – derived from plants, animals and microbes. One recent and important trend has been towards large-scale studies, in particular within biomedical and toxicological metabolic profiling [1-3]. While the necessary automation of data generation to support such large-scale studies is occurring [4, 5], and the automation of data

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https://doi.org/10.1016/j.aca.2018.03.019

processing workflows is increasingly being established [6, 7], the extraction of metabolites from biological samples remains a largely manual bottleneck in the metabolomics pipeline [8]; it is both a challenge for large-scale biofluid studies and an unsolved problem for studies of tissues, particularly the low-mass. High analytical reproducibility and throughput of the sample preparation step are crucial factors when measuring the metabolite compositions of hundreds to thousands of samples; however this is difficult to achieve using laborious manual extraction protocols. With advances in robotic technologies, automated sample handlers have the potential to replace manual sample processing in metabolomics [9], promising to open new horizons for large-scale studies.

The appropriate selection of extraction solvents has been a focus of the metabolomics community for several years [10-14]. Multiple factors should be considered: from maximising the chemical space of metabolites that are extracted through to maximising its operational simplicity, efficiency, reproducibility, speed and safety. The method first proposed by [15], which was originally intended to extract lipids, has proven so successful that it has been adopted by multiple laboratories worldwide [8, 16, 17]. This method utilises a chloroform/methanol/water (2/2/1.8, v/v/v) biphasic solvent system to extract both polar (methanol/water phase) and non-polar (chloroform phase) compounds separately. The extraction efficiency of chloroform stems from its ability to associate with water molecules through weak hydrogen bonds [18]. However, this solvent system has drawbacks, not least that chloroform is a carcinogen. Furthermore, the biphasic extraction results in a layer of protein and cellular debris between the upper polar and lower non-polar phases (called the interphase), which hinders the clean aspiration of the lower phase. While this is a known difficulty for manual liquid:liquid extractions, it represents a particular challenge for automated extractions using a liquid handling robot.

Significant efforts have been devoted to find an alternative to the Bligh and Dyer method such as hexane/isopropanol – 3/2, v/v [19], dichloromethane/methanol – 2/1, v/v [20], and hexane/ethanol – 5/2, v/v [21]. None of them, however, were reported to surpass the Bligh and Dyer method in terms of extraction efficiency. More recently, the Matyash method [22] was reported, which claimed to be at least as efficient as the chloroform/methanol/water method, and benefited from replacing chloroform by methyl-tert-butyl ether (MTBE), which is non-carcinogenic. The Matyash method (MTBE/methanol/water) utilises a non-polar phase (largely MTBE) that has a lower density than the methanol/water phase and hence it partitions on the top of the biphasic solvent system; this allows easier recovery of the lipid layer but correspondingly more difficult removal of the polar layer. More importantly – in terms of automation compatibility – the protein and cell debris layer is forced to the bottom of the sample tube following centrifugation, simplifying the removal of both solvent phases during the extraction. The Matyash method has been evaluated in animal [23-25] and plant samples [26, 27], proving its efficiency. The original method, however, is primarily focused on lipid extraction and unlike the Bligh and Dyer method has not been optimised for the recovery of both polar and non-polar metabolites from low-mass samples [8].

Here we have studied the extraction of two biofluids (human plasma and urine) and one tissue type (whole water flea *Daphnia magna*) in order to select a metabolite extraction protocol that offers superior metabolite yield and reproducibility and provides the highest benefit for automation (in terms of method duration, use of resources, simplicity to automate). We compare the gold-standard Bligh and Dyer extraction method (chloroform/methanol/water, stepwise) to two variations of the Matyash method (MTBE/methanol/water) – the original published protocol (MTBE/methanol/water, 10/3/2.5, v/v/v) and a modified method (MTBE/methanol/water, 2.6/2.0/2.4, v/v/v) – the latter employs solvent ratios that match those used by the Bligh and Dyer method and thereby increase the volume of the polar phase for easier handling. Specifically, we compare the extraction yields, derived from measurements of the number of peaks and putatively annotated metabolites detected in ultra performance liquid chromatography-mass spectrometry (UHPLC-MS) and direct infusion mass spectrometry (DIMS), and extraction reproducibilities, calculated as the median relative standard deviation – mRSD [28] of all detectable metabolites.

## 2. Materials and methods

### 2.1. Biological samples

Three well studied yet diverse sample types were selected to ensure our results are widely applicable: two human biofluids, plasma and urine, and a toxicological and US National Institutes of Health model organism (*D. magna*). Biofluids (100 μl aliquots from pooled frozen samples) were acquired from Sera Laboratories International Ltd (West Sussex, UK). *D. magna* was cultured in OECD media, fed on *Chlorella sp.*, and <24 h neonates (30 animals per sample, flash frozen in liquid nitrogen and stored at −80 °C) were used for experiments [29].

### 2.2. Metabolite extraction methods

Three extraction protocols were compared – Bligh and Dyer method (chloroform/methanol/water, 2/2/1.8, v/v/v) as optimised for metabolomics studies of tissues [8], the original Matyash method (MTBE/methanol/water, 10/3/2.5, v/v/v; [22]) and our modification of the Matyash method (MTBE/methanol/water, 2.6/2.0/2.4, v/v/v) to use solvent ratios and volumes that were equivalent to the successful Bligh and Dyer method and that were compatible with the automated extraction of both the polar and non-polar phases. Biofluids were extracted using the same protocols, however, without homogenization. Each method is described in more detail in the following sections. For each sample type-extraction method combination, 10 replicates were used.

### 2.3. Bligh and Dyer (stepwise) method

As described by Wu et al. [8], with some minor changes, first 75% ice cold methanol (32 $\mu l\ mg^{-1}$ methanol and 10.6 $\mu l\ mg^{-1}$ or 0.9 $\mu l\ mg^{-1}$ HPLC water for tissues and biofluids, respectively) was added to samples and they were homogenised (tissue only) in a Precellys-24 bead-based homogeniser (Bertin technologies) for 2 × 10s bursts at 6400 rpm. Homogenates were each transferred into 1.8 ml glass vials and 16 $\mu l\ mg^{-1}$ (or 2 $\mu l\ mg^{-1}$ for biofluids) of chloroform was added. Samples were mixed using a Bioshake platform (2000 rpm, 3 min; Bioshake 3000 elm (Edge Locking Mechanism), Quantifoil Instruments GmbH) and then centrifuged (2415 × g, 10 min, 18 °C; refrigerated centrifuge 6-16KR, Sigma) to pellet the protein and tissue debris. Each monophasic supernatant (~500 μl) was transferred to a clean 1.8 ml glass vial and phase separation was induced by adding 16 $\mu l\ mg^{-1}$ (or 2 $\mu l\ mg^{-1}$ for biofluids) of chloroform and 18.2 $\mu l\ mg^{-1}$ (or 2.27 $\mu l\ mg^{-1}$ for biofluids) of HPLC water. Samples were then mixed again on the Bioshake (2000 rpm, 1 min), incubated at 18 °C for 10 min to allow the partitioning of the solvent system and then centrifuged (2415 × g, 10 min, 18 °C). Polar and non-polar fractions were aliquoted into clean Eppendorf tubes or glass vials, respectively, and then dried down using a SpeedVac concentrator (SPD111V, Thermo Savant; for polar samples only) or nitrogen blow-down evaporator (TECHNE sample concentrator with Peak Scientific Genius nitrogen

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generator; for non-polar samples only).

### 2.4. Matyash method

This method followed the original Matyash extraction [22] with some minor modifications in order to make all methods comparable. Samples were homogenised (tissue only) in 75% cold methanol as in section 2.3 and homogenates were transferred into 1.8 ml glass vials. Then 107 μl $mg^{-1}$ (or 13.3 μl $mg^{-1}$ for biofluids) of MTBE was added to each vial and the samples were mixed on the Bioshake (2000 rpm, 3 min). Phase separation was then induced by adding 16.1 μl $mg^{-1}$ (or 2 μl $mg^{-1}$ for biofluids) of HPLC water. Samples were then mixed again on the Bioshake (2000 rpm, 1 min), incubated at 18 °C for 10 min to allow phase separation to begin, and centrifuged (2415 × g, 10 min, 18 °C). Polar and non-polar fractions were aliquoted into clean Eppendorf tubes or glass vials, respectively, and dried as in 2.3.

### 2.5. Modified Matyash method

The solvent ratios used in this method were customised to match those for the Bligh and Dyer protocol. This method was identical to the Bligh and Dyer (stepwise), until the tissue homogenates were transferred into 1.8 ml glass vials. MTBE (16 μl $mg^{-1}$ or 2 μl $mg^{-1}$ for tissues and biofluids, respectively) was then added and samples were mixed on a Bioshake (2000 rpm, 3 min). Phase separation was induced by adding 25.6 μl $mg^{-1}$ (or 3.2 μl $mg^{-1}$ for biofluids) of MTBE and 27.8 μl $mg^{-1}$ (or 3.47 μl $mg^{-1}$ for biofluids) of HPLC water. Samples then were mixed on the Bioshake (2000 rpm, 1 min), incubated at 18 °C for 10 min, and centrifuged (2415 × g, 10 min, 18 °C). Polar and non-polar fractions were aliquoted and then dried down as described in section 2.3.

### 2.6. Direct infusion mass spectrometry

Due to a low sample biomass and the superior sensitivity of the method [29], *D. magna* samples (only) were analysed using SIM-stitch direct infusion mass spectrometry (DIMS), as reported previously [30]. The rest of the sample types were analysed using (UHPLC-MS). Dried polar extracts were resuspended in 80/20 methanol/water (HPLC grade) containing 0.25% formic acid for positive ion mass spectrometry, or 80/20 methanol/water containing 20mM ammonium acetate for negative ion analysis. Non-polar extracts were resuspended in 2/1 methanol/chloroform with 5mM ammonium acetate. Samples were analysed using a Quadrupole-Orbitrap mass spectrometer (Q-Exactive, Thermo Fisher Scientific, Hemel Hempstead, UK) equipped with a chip-based direct infusion nano-ESI (electrospray ionisation) assembly (Triversa, Advion Biosciences, Ithaca, NY, USA). Nano-ESI conditions consisted of 0.3 psi backing pressure and 1.4 kV or −1.4 kV electrospray voltage (for positive and negative ion analysis, respectively), all controlled by ChipSoft software (version 8.3.3, Advion Biosciences). The mass range was from 100 to 1000 Daltons (Da).

### 2.7. Ultra performance liquid chromatography – mass spectrometry

Dried polar and non-polar extracts were resuspended as above. Samples were analysed using a Quadrupole-Orbitrap mass spectrometer (Q-Exactive) equipped with a Dionex UltiMate 3000 UHPLC (ultra high performance liquid chromatography; Thermo Fisher Scientific) employing reverse phase and HILIC (hydrophilic interaction liquid chromatography) methods. For the reverse phase analyses, solvents were composed of 0.1% formic acid in HPLC water (A) and 0.1% formic acid in methanol (B). For HILIC, the solvents contained 95% acetonitrile with the addition of 5 mM ammonium formate (pH = 3; A) and HPLC water with 5mM ammonium formate (pH = 3; B). Ion source conditions comprised: spray voltage −3.5kV (for negative mode) and +4.5kV (for positive mode); resolution of 70,000 (for single polarity mode) and 35,000 (for polarity switching mode). The mass spectral range was from 100 to 1000 Da.

### 2.8. Data processing and analysis

SIM-stitch DIMS data comprised of a series of narrow overlapping mass windows that were processed as described previously using custom-written Matlab software [30, 31]. In brief, only peaks with a signal to noise ratio greater than 10, that passed the 'replicate filter' (i.e. present in at least 2 of the 3 technical replicates per sample), passed the blank filter (i.e. present in the biological samples with at least ten times the intensity of a potential occurrence in the blank samples, designed to remove solvent and contaminant peaks from the dataset), and that passed the '80% sample filter' by class (i.e. present in at least 80% of the biological replicates of any one extraction method) were retained in the datasets. UHPLC-MS data were processed applying the freely available software XCMS, operated in R applying previously defined parameters [32]. Data acquired applying DIMS and UHPLC-MS were normalized applying probabilistic quotient normalisation (PQN) and SUM normalisation, respectively. Relative standard deviation (RSD) of the peak intensities was calculated across all the detected peaks and a single median RSD was determined for each extraction method and ion mode combination, where the median RSD value provides a measure of the reproducibility of that dataset [28]. It is important to note that for each biofluid a pooled sample was investigated such that any variability in the mass spectra would be known to arise from the sample preparation and LC-MS measurements. For *Daphnia*, each sample investigated was a different pool of 30 animals; hence while that biological pooling of animals (per sample) greatly reduced the biological variability between the samples, the variability in the mass spectra could arise from slight biological differences between samples or from the metabolite extraction and DIMS measurements. Peak counts (total number of peaks for each of the three extraction methods), the corresponding *m/z* values (DIMS) and *m/z*-retention time (rt) pairs (UHPLC-MS), and peak intensities were extracted from the Matlab software. DIMS data were then putatively annotated using MI-Pack v2_01 software (based on Python 27) employing KEGG database and LIPIDMAPS for polar and non-polar compounds, respectively (with the maximum mass error of 3 ppm). LC-MS data were putatively annotated employing PUTMEDID operated in the Taverna workflow environment applying standard parameters [33]. Venn diagrams were created using Bioinformatics and Evolutionary Genomics website tool at http://bioinformatics.psb.ugent.be/webtools/Venn/.

## 3. Results and discussion

### 3.1. Extraction yield

First, all extraction strategies were compared based upon the number of peaks detected, for each sample type and for each mass spectrometry ion mode (Fig. 1). In addition, Fig. 1 shows which peaks are common across two or three of the extraction strategies. Overall, the modified Matyash method yielded more peaks in biofluids than both the original Matyash method (1–29% more; except urine non-polar samples, where original Matyash yielded 4–7% more peaks) and the Bligh and Dyer method (4–20% more; only in non-polar negative plasma samples equal number of peaks was generated) when combining results for the upper and lower phases

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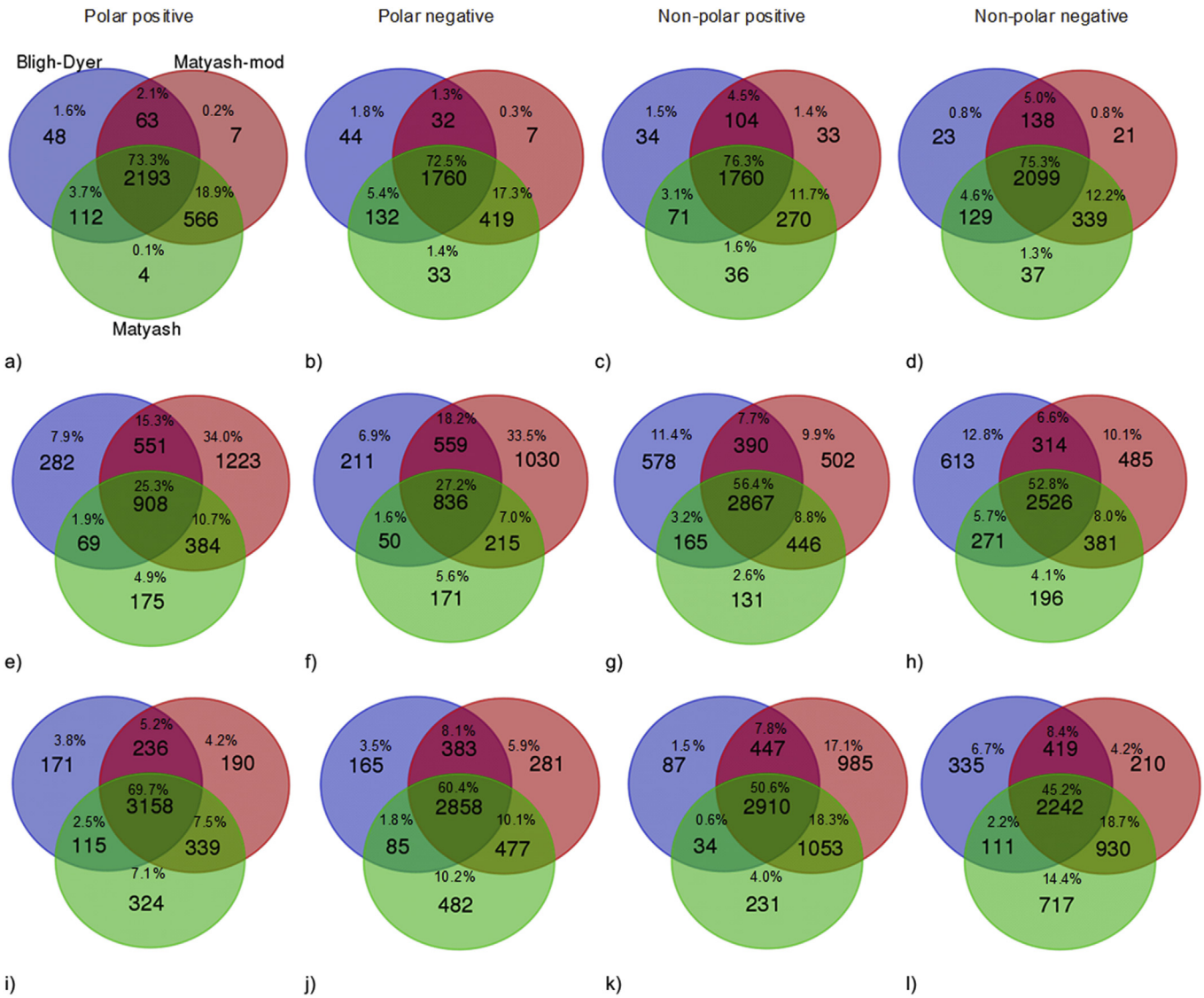


**Fig. 1.** Extraction method comparison based on peak counts as a surrogate measure of metabolite yield. Venn diagrams represent the numbers of unique and common peaks (and relative percentages) between the three extraction methods: stepwise Bligh and Dyer – blue circle, Matyash modified – red circle, and original Matyash – green circle) in a-d) *D. magna*, e-h) human plasma, and i-l) human urine (n = 10), for positive (a, e, i) and negative (b, f, j) ion analysis of polar metabolites, and positive (c, g, k) and negative (d, h, l) ion analysis of non-polar metabolites.

(Table A.1). It also yielded more unique peaks (peaks that were detected in only one out of three methods) than these two other extraction methods. In *D. magna* samples on the other hand the original Matyash method performed better than the other two (yielding 1–5% more peaks than modified Matyash and 7–15% more than the Bligh and Dyer method).

Specifically, in *D. magna* samples (Fig. 1 (**a**–**d**)), the original and modified Matyash methods showed very similar peak counts both in the polar and non-polar phases with minimal unique peaks. The Bligh and Dyer protocol performed slightly poorer (yielding 6–16% fewer peaks), however generated the majority of unique peaks. While some clear differences exist between the methods, between 73 and 76% of the peaks detected were extracted by all three protocols, highlighting the relative consistency of biphasic extractions and DIMS analyses of this tissue type. In plasma samples (Fig. 1 (e–h)) the modified Matyash method showed superior results yielding 27–29% and 16–20% more polar peaks than the original Matyash and Bligh and Dyer methods, respectively (except for the analyses of the non-polar fractions (Fig. 1 (g, h)) for which the Bligh and Dyer method detected a comparable number of peaks to the modified Matyash protocol). In urine samples (Fig. 1 (i–l)) the original Matyash method yielded the highest number of peaks (4–7% and 14–18% more non-polar peaks than the modified Matyash and Bligh and Dyer methods, respectively). In summary, the original Matyash method showed superior extraction yield in *D. magna* and urine samples (except for polar negative); the modified Matyash method yielded a comparable (to the original Matyash) number of peaks in *D. magna* and polar urine samples, and was superior to the other two methods for non-polar plasma samples; and the Bligh and Dyer method was comparable to the modified Matyash method for non-polar plasma samples.

Furthermore, extraction methods were also compared based on putatively annotated metabolites, for each sample type and ion mode (Fig. 2). In general, and as anticipated, trends for metabolites were consistent with trends for the number of peaks detected; the modified Matyash method again performed better in biofluids than

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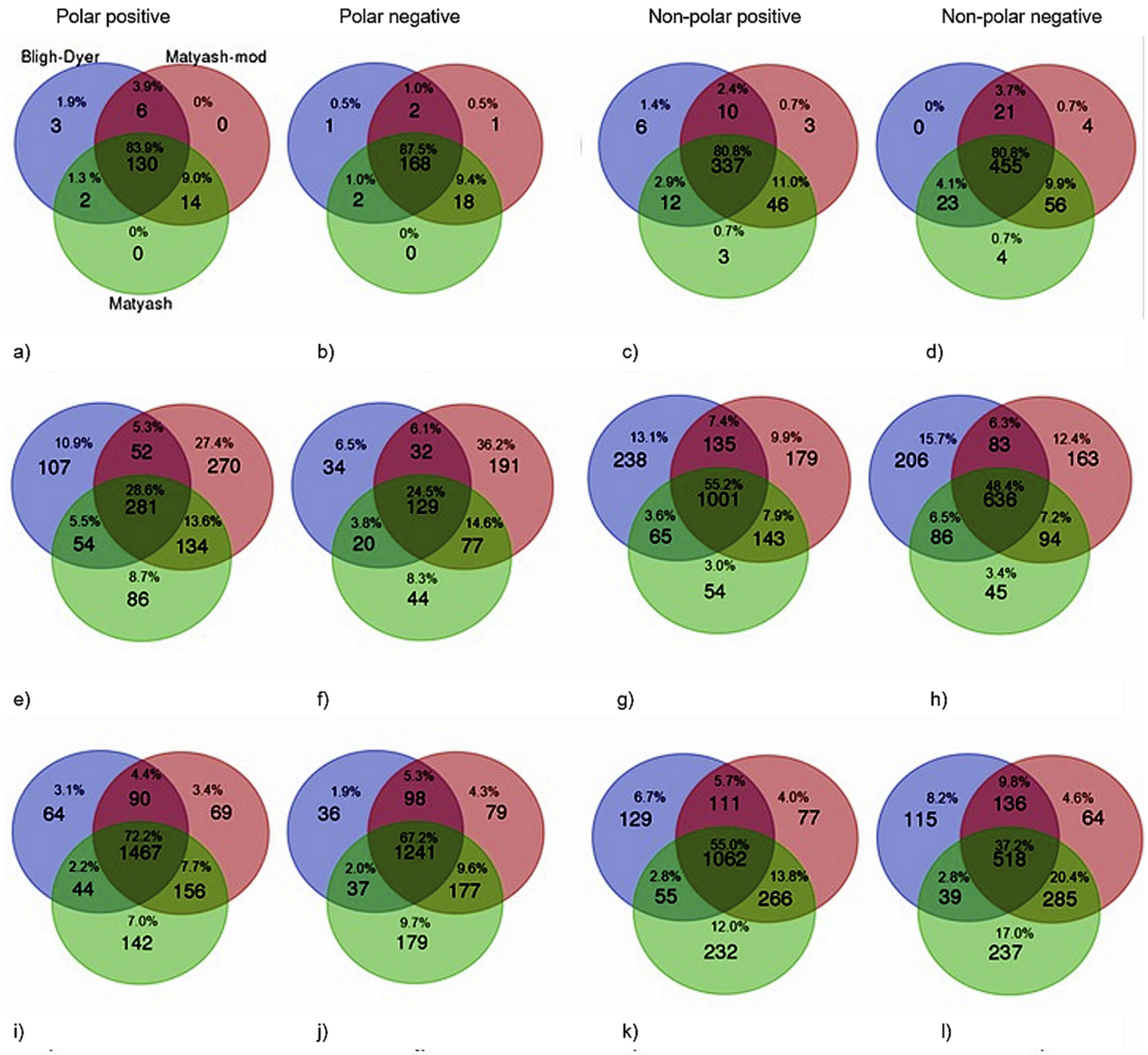


**Fig. 2.** Extraction method comparison based on number of putatively annotated metabolites. Venn diagrams represent the numbers of unique and common metabolites (and relative percentages) between the three extraction methods: stepwise Bligh and Dyer – blue circle, Matyash modified – red circle, and original Matyash – green circle) in a-d) *D. magna*, e-h) human plasma, and i-l) human urine (n = 10), for positive (a, e, i) and negative (b, f, j) ion analysis of polar metabolites, and positive (c, g, k) and negative (d, h, l) ion analysis of non-polar metabolites.

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the original Matyash method (with 11–30% more annotated metabolites in plasma, and approximately the same number in urine samples) and the Bligh and Dyer method (1–41% more annotated metabolites; similar number of metabolites were detected in non-polar negative plasma samples) when combining results for the polar and non-polar metabolites (Table A.2). In *D. magna* samples the original and modified Matyash method yielded almost equal numbers of metabolites, whereas the Bligh and Dyer method yielded 3–8% less metabolites.

Specifically, the original and modified Matyash methods performed equally in both polar (only in polar positive samples did the modified Matyash perform slightly better and yielded 3% more metabolites) and non-polar phases, both surpassing the Bligh and Dyer protocol (recovering 3–8% more metabolites in polar and 7–8% in non-polar samples) in *D. magna* samples (Fig. 2 (a–d)). Similar to the peak counts, all three extraction methods proved to be highly consistent, as 81–88% of the annotated metabolites were present in all three datasets. In plasma samples (Fig. 2 (e–h)) the modified Matyash method yielded 9–30% and 1–41% more metabolites than the original Matyash and Bligh and Dyer methods, respectively (except for non-polar negative samples (Fig. 2 (h)) for which the Bligh and Dyer method detected 3% more metabolites). In urine samples (Fig. 2 (i–l)) the original Matyash method was superior compared to the other two methods (yielding 1–6% and 7–19% more metabolites than the modified Matyash and Bligh and Dyer methods, respectively). Overall, the modified Matyash method was superior in *D. magna* polar and plasma samples, and comparable (to the original Matyash) in *D. magna* non-polar samples; the original Matyash method showed higher metabolite yield in urine samples; and the Bligh and Dyer method was comparable to the modified Matyash method for non-polar plasma samples.

Several non-polar (putatively annotated) compounds were selected from various lipid groups (including phosphatidic acids - PA, phosphatidylcholines - PC, phosphatidylethanolamines - PE, phosphatidylglycerols - PG, phosphatidylinositols - PI, phosphatidylserines - PS, sphingomyelins – SM and triglycerides - TG) and compared across the three extraction strategies using PQN normalized signal intensities (Fig. A2). In general, in *D. magna* samples (only 2 metabolites had lower intensities in modified Matyash method) as well as in plasma (only 4 metabolites showed significantly lower intensities in Bligh and Dyer compared to the other two methods), all three methods showed very similar lipid intensities. In urine samples, however, the modified Matyash and Bligh and Dyer methods showed similar intensities whereas the original Matyash intensities were lower (for 11 out of 17 metabolites significantly lower than both other methods, and for 2 metabolites lower than modified Matyash).

In addition, all putatively annotated amino acids in each sample type were selected in order to evaluate and compare polar metabolite intensities (Fig. A3). In *D. magna* polar samples both the original and modified Matyash methods performed equally well, surpassing the Bligh and Dyer method (showing significantly higher metabolite intensities in 14 out of 24 amino acids; the remaining 10 were similar intensity across all three methods). In plasma and urine, however, the majority of amino acid intensities were similar across all three methods (only 3 metabolites had lower intensities in the original Matyash method compared to the other two).

To date, numerous studies have investigated the sample extraction efficiencies of the Bligh and Dyer or Folch methods [34]. Recently, the Matyash method has gained considerable popularity though most of the studies have focused on the extraction of metabolites from biofluids, in particular plasma. For instance, Heiskanen et al. [35] used a Folch extraction and shotgun lipidomics approach to measure lipids in human plasma, recovering 610 and 639 lipids in negative and positive ion modes, respectively. Jung et al. [36] also used the Folch method to perform high-throughput molecular lipidomics in plasma and quantified several hundreds of lipids. Few studies have compared the Matyash method to the long-standing Bligh and Dyer and Folch methods in plasma samples. In one study [24] all three methods (Matyash, Bligh and Dyer, Folch) as well as two global metabolite extraction methods for both lipids and polar metabolites using DIMS and GC-MS were examined and evaluated. The authors concluded that the Folch and Matyash methods performed equally well when assessing lipids (in terms of the number of metabolites extracted); while for the extraction of polar metabolites the acetonitrile /isopropanol/water (3/3/2, v/v/v) method was recommended. Another study [37] also compared the Matyash and Folch methods (in plasma), employing LC-MS, and concluded that both extracted lipids equally well, however Matyash surpassed the Folch method when extracting polar metabolites; therefore they recommended the Matyash method for untargeted metabolomics and lipidomics. Yang et al. [38] optimised the Matyash method and compared it to a simple methanol precipitation protocol, using LC-MS to show that the new Matyash method recovered 3806 versus only 1851 metabolic features in plasma. Furthermore, Whiley et al. [39] also modified the Matyash method employing it in so called in-vial dual extraction (IVDE) of plasma, and analysed the samples via HPLC-QTOF MS, recovering over 4500 features.

Several studies have investigated metabolite extraction strategies from urine. For instance, Bang et al. [40] compared the Folch and Matyash methods using nanoflow LC-ESI–$MS^3$ and concluded that the Folch was superior when extracting different phospholipid classes in negative ion mode. Another study [41] showed that HPLC water combined with pre-concentration by solid-phase extraction (SPE) retained 3503–4484 peaks (in positive and negative modes, respectively) using nanoflow UHPLC-nanoESI-TOF-MS. Most of these studies are in agreement with our results, concluding that the Matyash method surpasses both the Folch and Bligh and Dyer methods in terms of peak recovery from biofluids.

Various studies have focused on metabolite extraction of animal tissues (primarily of dissected organs) predominantly employing either the Bligh and Dyer or Folch methods, however only a few have investigated metabolite extraction in the whole organism. Taylor et al. [29] showed that *D. magna* extracted using the Bligh and Dyer method yields 1848 and 3599 polar features (positive and negative ion modes, respectively) following DIMS analysis. No earlier studies have compared the extraction yields of the Matyash and Bligh and Dyer methods in tissue samples. Here we have reported evidence, consistent with earlier studies of biofluids, that the Matyash (or modified Matyash) method is superior (recovering 5–20% more peaks) or comparable (in plasma non-polar samples) to the Bligh and Dyer method.

### 3.2. Reproducibility

Reproducibility of the extraction methods was compared based on the relative standard deviations (RSDs) of the intensities of the peaks (Fig. 3), in particular the median of these values (mRSD; also termed coefficient of variance) which we have shown previously to serve as a useful statistic for benchmarking the reproducibility of methods [28]. Overall, the median RSD values of the modified Matyash method were lower than those for the Bligh and Dyer and

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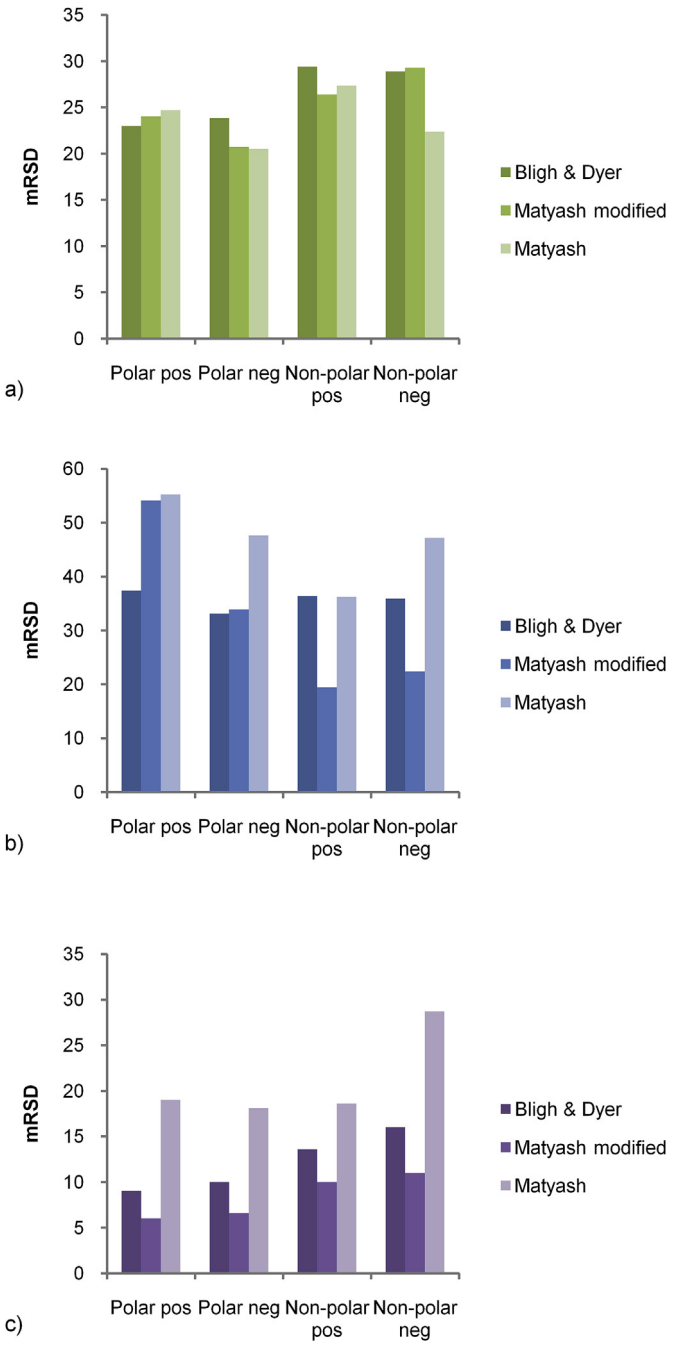


**Fig. 3.** Extraction method comparison based on the reproducibility of the metabolic peak intensities. Bar charts represent mRSD of each of the three extraction methods (stepwise Bligh and Dyer, modified and original Matyash) for the analysis of polar and non-polar metabolites (pos. – positive and neg. – negative ion modes) in a) *D. magna*, b) human plasma, and c) urine (n = 10).

original Matyash methods, across different sample types and polar/non-polar datasets (Fig. A1).

Specifically, the reproducibility of *D. magna* metabolomics data (Fig. 3 (a)) was very similar across all methods, for polar (with mRSD 20–24%) and non-polar (mRSD – 22–29%) metabolites. RSD values over 20% are not unexpected as the variation across these samples could predominantly arise from biological differences (each sample comprises of a pool of 30 individual organisms) rather than differences introduced through the sample preparation. The trends in mRSD values were less clear in plasma (Fig. 3 (b)), although the modified Matyash datasets showed the lowest values (19–33%, except positive ion data of polar metabolites with mRSD of 54%), followed by Bligh and Dyer (33–37%) and then the original Matyash method with surprisingly high metabolic variation (36–55%). In urine samples (Fig. 3 (c)), the modified Matyash method again performed better (with mRSD 6–11%) than both other methods (mRSD values of 9–16% and 18–28% for Bligh and Dyer and the original Matyash method, respectively). In summary, all three methods showed similar reproducibility in *D. magna* potentially due to the confounding factor of biological variability, while the reproducibility of the modified Matyash method was superior in urine and non-polar plasma samples, and the Bligh and Dyer method showed higher reproducibility in polar plasma samples.

Various studies of plasma have reported high sample reproducibility for the Folch, Bligh and Dyer and Matyash methods, generally with mRSD below 15% [35]. Lee et al. [24] stated that reproducibility of Bligh and Dyer method (mRSD of 18%) was lower than Matyash method (mRSD of 12%), whereas another study [37] indicated that reproducibility of the Folch and Matyash methods was equally high. Yang et al. [38] showed that in plasma samples the Matyash method RSDs generally were below 15% while extraction using methanol precipitation led to RSDs of 30%. The reproducibility of these results is comparable with modified Matyash method in non-polar plasma fractions; polar samples however, showed surprisingly high mRSD values in all three methods tested. On the contrary, in urine samples the modified Matyash method showed excellent reproducibility of polar fractions (as well as non-polar, with mRSDs below 11%), consistent with the previous biofluid studies. Furthermore, in *D. magna* samples [29] mRSD values of polar fractions extracted using Bligh and Dyer were calculated and the reported value was <25% for 20–50 neonates, consistent with the current study.

## 4. Conclusions

In this study we have compared a Bligh and Dyer extraction to both the original and a modified Matyash method, across three distinct sample types, and compared the metabolite yields and reproducibility using DIMS and UHPLC-MS analyses. The aim of this study was to determine which extraction method provided the highest metabolite yield and reproducibility, and which would be the most suitable for automation on a liquid handling robot. We have shown that the modified Matyash method – which was based on the original Matyash method, but with solvent volumes more similar to the Bligh and Dyer protocol – has a higher extraction yield as it typically recovers the largest number of peaks (apart from urine non-polar and *D. magna* samples, where it is comparable) in both polar (5–29%) and non-polar (1–14%) fractions, as well as the largest number of putatively annotated metabolites (apart from urine samples) in polar (1–41%) and non-polar (1–14%) samples, compared with the other two methods tested. It also provided superior or comparable metabolite intensities in both polar (while Bligh and Dyer showed significantly lower intensities in *D. magna*) and non-polar (original Matyash performed significantly poorer in urine samples) annotated metabolites. Furthermore, we have demonstrated that the reproducibility of the modified Matyash method is higher or comparable to the Bligh and Dyer and original Matyash methods (excluding polar plasma samples). Collectively this provides evidence for the use of this less toxic modified Matyash method, for the majority of sample types

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tested herein, rather than the traditional Bligh and Dyer extraction. Furthermore, this study serves as a basis for the development of automated sample extraction methodologies.

## Declaration of interests

None.

## Funding

We thank the UK Natural Environment Research Council and UK Biotechnology and Biological Sciences Research Council for PhD studentships for JS (NE/L009102/1) and KC (BB/L015579/1), respectively, and Thermo Fisher Scientific for two CASE awards.

## Acknowledgements

We would like to thank Drs. Martin Jones and Ralf Weber for their help in metabolite annotation.

## Appendix

**Table A.1**
Extraction method comparison based on peak counts across the three methods tested. Table shows extraction yield (number and percentage of peaks recovered) of three methods (stepwise Bligh and Dyer, modified and original Matyash) in 3 sample types (*D. magna*, human plasma, and human urine).

| Tissue | Polarity | Ion mode | Total peak number* | Extraction yield, number of peaks | | | Extraction yield, % of total peaks recovered | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Bligh & Dyer | Modified Matyash | Matyash | Bligh & Dyer | Modified Matyash | Matyash |
| *D. magna* | Polar | Positive | 2993 | 2416 | 2829 | 2875 | 80.72 | 94.52 | 96.06 |
| | | Negative | 2428 | 1968 | 2218 | 2344 | 81.05 | 91.35 | 96.54 |
| | Non-polar | Positive | 2308 | 1969 | 2167 | 2137 | 85.31 | 93.89 | 92.59 |
| | | Negative | 2786 | 2389 | 2597 | 2604 | 85.75 | 93.22 | 93.47 |
| Plasma | Polar | Positive | 3592 | 1810 | 2522 | 1536 | 50.39 | 70.21 | 42.76 |
| | | Negative | 3072 | 1656 | 2169 | 1272 | 53.91 | 70.61 | 41.41 |
| | Non-polar | Positive | 5079 | 4000 | 4205 | 3609 | 78.76 | 82.79 | 71.06 |
| | | Negative | 4786 | 3724 | 3706 | 3374 | 77.81 | 77.43 | 70.50 |
| Urine | Polar | Positive | 4533 | 3680 | 3923 | 3936 | 81.18 | 86.54 | 86.83 |
| | | Negative | 4731 | 3491 | 3999 | 3902 | 73.79 | 84.53 | 82.48 |
| | Non-polar | Positive | 5747 | 3477 | 3854 | 4228 | 60.50 | 67.06 | 73.57 |
| | | Negative | 4964 | 3107 | 3801 | 4000 | 62.59 | 76.57 | 80.58 |

*Total number of peaks extracted by all three methods

**Table A.2**
Extraction method comparison based on number of putatively annotated metabolites across the three methods tested. Table shows number and percentage of metabolites putatively annotated in three methods (stepwise Bligh and Dyer, modified and original Matyash) in 3 sample types (*D. magna*, human plasma, and human urine).

| Tissue | Polarity | Ion mode | Total metabolite number | Number of annotated metabolites | | | % of metabolites annotated | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Bligh & Dyer | Modified Matyash | Matyash | Bligh & Dyer | Modified Matyash | Matyash |
| *D.magna* | Polar | Positive | 155 | 141 | 150 | 146 | 90.97 | 96.77 | 94.19 |
| | | Negative | 192 | 173 | 189 | 188 | 90.10 | 98.44 | 97.92 |
| | Non-polar | Positive | 417 | 365 | 396 | 398 | 87.53 | 94.96 | 95.44 |
| | | Negative | 563 | 499 | 536 | 538 | 88.63 | 95.20 | 95.56 |
| Plasma | Polar | Positive | 984 | 494 | 737 | 555 | 50.20 | 74.90 | 56.40 |
| | | Negative | 527 | 215 | 429 | 270 | 40.80 | 81.40 | 51.23 |
| | Non-polar | Positive | 1815 | 1439 | 1458 | 1263 | 79.28 | 80.33 | 69.59 |
| | | Negative | 1313 | 1011 | 976 | 861 | 77.00 | 74.33 | 65.58 |
| Urine | Polar | Positive | 2032.0 | 1665 | 1782 | 1809 | 81.94 | 87.70 | 89.03 |
| | | Negative | 1847.0 | 1412 | 1595 | 1634 | 76.45 | 86.36 | 88.47 |
| | Non-polar | Positive | 1932.0 | 1357 | 1516 | 1615 | 70.24 | 78.47 | 83.59 |
| | | Negative | 1394.0 | 808 | 1003 | 1079 | 57.96 | 71.95 | 77.40 |

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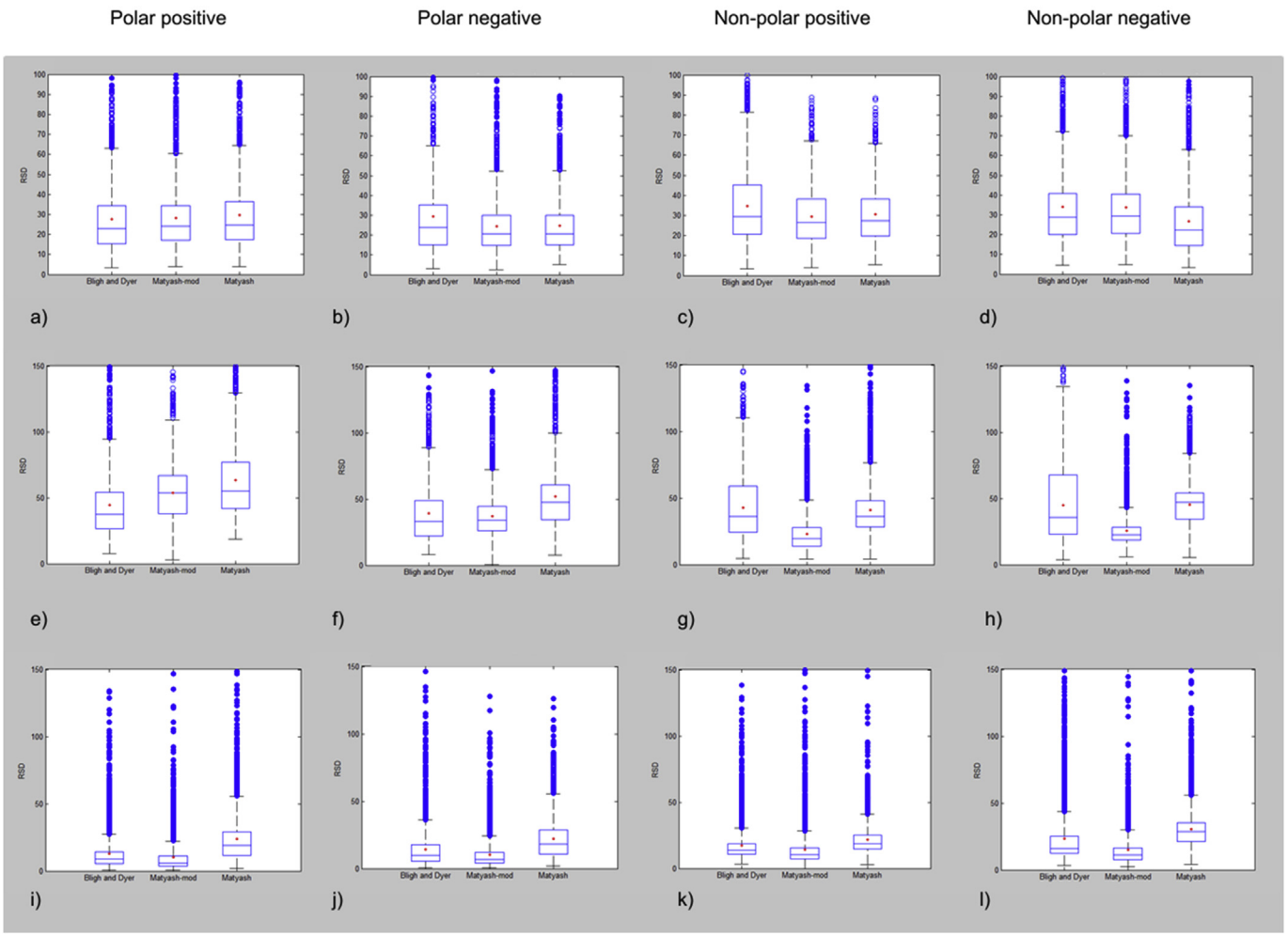


**Fig. A.1.** Extraction method comparison based on the reproducibility of peak intensities. Box plots represent mRSD (shown as a horizontal line within each box) for the three extraction methods (stepwise Bligh and Dyer, modified and original Matyash) in a-d) *D. magna*, e-h) human plasma, and i-l) urine, for positive (a, e, i) and negative (b, f, j) ion analysis of polar metabolites, and positive (c, g, k) and negative (d, h, l) ion analysis of non-polar metabolites. The outliers are shown as filled blue circles and comprise <10% of each dataset.

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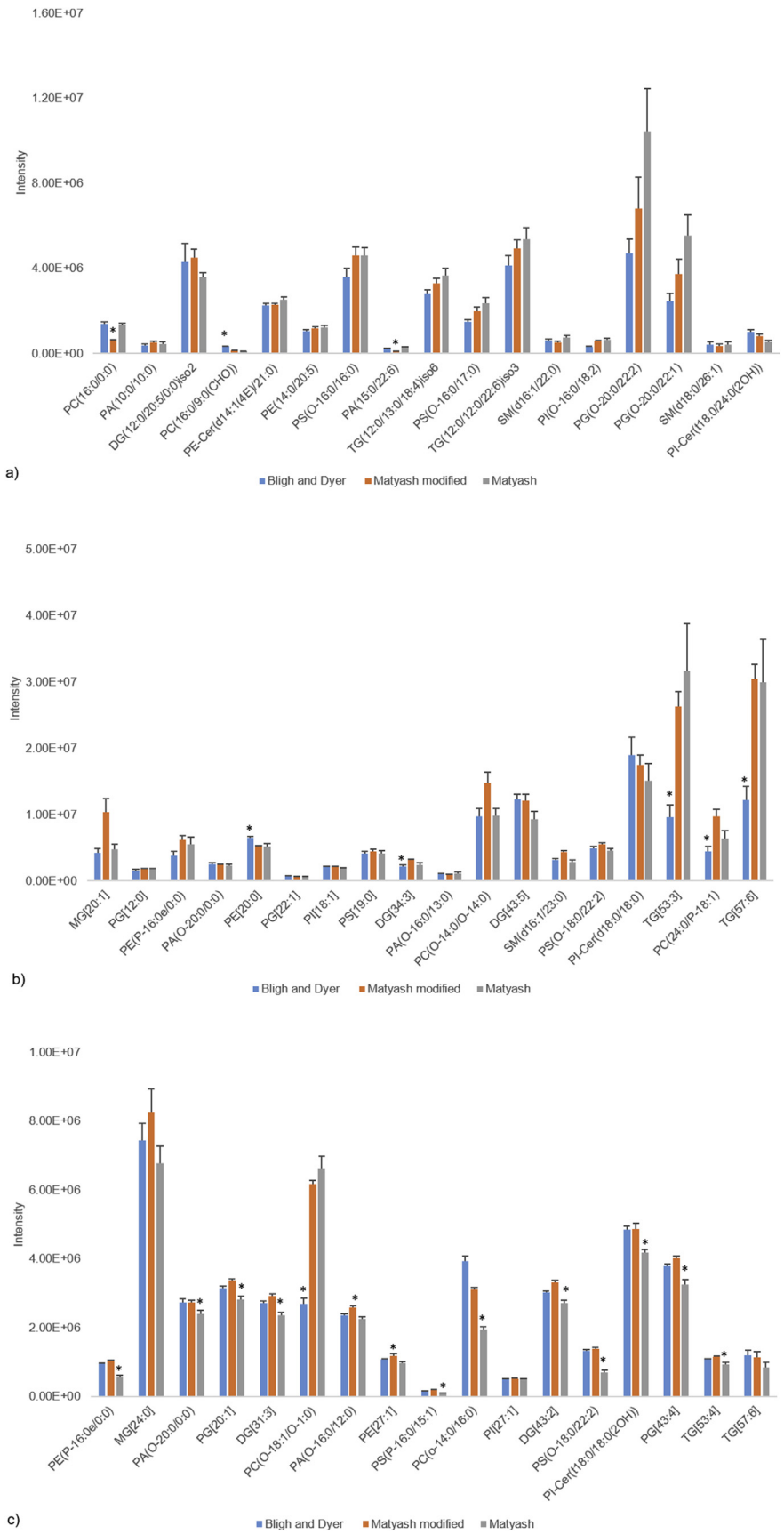


**Fig. A.2.** Comparison of intensities of non-polar metabolites extracted from three sample types using three different methods. Blue bars represent Bligh and Dyer method, orange - Matyash modified, and grey - original Matyash methods in a) *D. magna*, b) human plasma, and c) human urine (n = 10). Error bars show the standard error. Stars highlight significant changes in intensity ($p < 0.05$). More details on the metabolites can be found in Table A.3-A.5.

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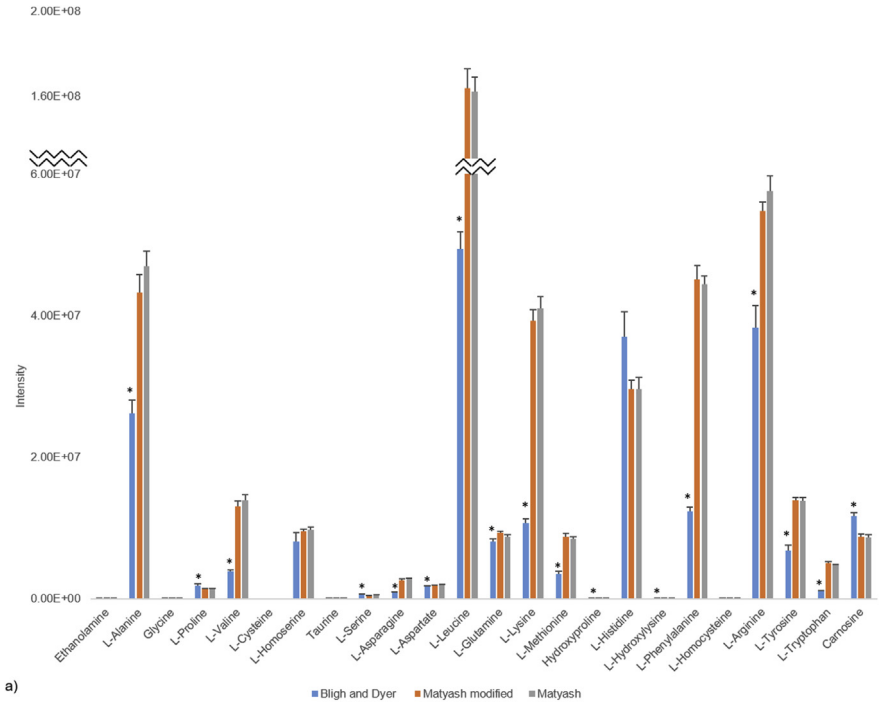
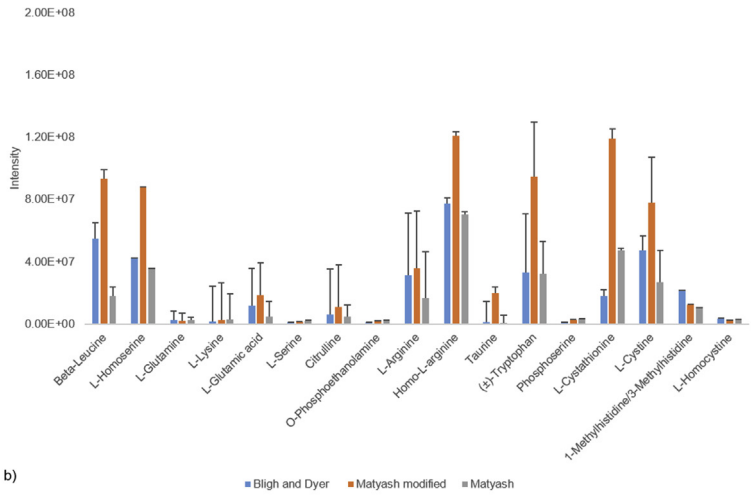
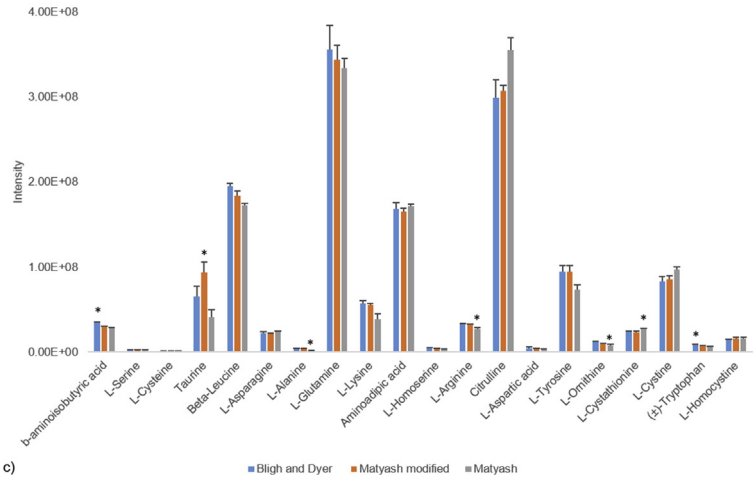



a)




b)




c)

**Fig. A.3.** Comparison of intensities of amino acids extracted from three sample types using three different methods. Blue bars represent Bligh and Dyer method, orange - Matyash modified, and grey - original Matyash methods in a) *D. magna*, b) human plasma, and c) human urine (n = 10). Error bars show standard error. Stars highlight significant change in intensity ($p < 0.05$). More details on the metabolites can be found in Table A.6-A.8.

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**Table A.3**
MI-Pack annotation of the non-polar peaks detected in *D. magna* via DIMS and presented in Fig. A2 (a).

| *m/z* | Empirical formula | Ion form | Theoretical mass (Da) | Mass error (ppm) | Metabolite name |
|---|---|---|---|---|---|
| 496.3399 | C24H50NO7P | [M+H]+ | 495.3325 | 0.22 | ['PC(0:0/16:0)', 'PC(16:0/0:0)', 'PC(16:0/0:0)[rac]', 'PC(O-14:0/2:0)', 'PE(19:0/0:0)'] |
| 519.2492 | C23H45O8P | [M+K]+ | 480.2852 | 1.51 | ['PA(10:0/10:0)'] |
| 599.3904 | C35H58O5 | [M+(41K)]+ | 558.4284 | 1.18 | ['DG(12:0/20:5(5Z,8Z,11Z,14Z,17Z)/0:0)[iso2]'] |
| 650.4389 | C33H64NO9P | [M+H]+ | 649.4319 | −0.41 | [' PC(16:0/9:0(CHO))'] |
| 675.5438 | C37H75N2O6P | [M+H]+ | 674.5363 | 0.29 | ['PE-Cer(d14:1(4E)/21:0)', 'PE-Cer(d15:1(4E)/20:0)', 'PE-Cer(d16:1(4E)/19:0)', 'SM(d16:1/16:0)', 'SM(d18:1/14:0)'] |
| 710.4762 | C39H68NO8P | [M+H]+ | 709.4683 | 0.91 | ['PE(14:0/20:5(5Z,8Z,11Z,14Z,17Z))', 'PE(14:1(9Z)/20:4(5Z,8Z,11Z,14Z))', 'PE(16:1(9Z)/18:4(6Z,9Z,12Z,15Z))', 'PE(18:4(6Z,9Z,12Z,15Z)/16:1(9Z))', 'PE(20:4(5Z,8Z,11Z,14Z)/14:1(9Z))', 'PE(20:5(5Z,8Z,11Z,14Z,17Z)/14:0)'] |
| 760.4892 | C38H76NO9P | [M+K]+ | 721.5258 | 0.39 | ['PS(O-16:0/16:0)', 'PS(O-18:0/14:0)', 'PS(O-20:0/12:0)'] |
| 765.4705 | C40H67O8P | [M + Hac-H]- | 706.4574 | −0.98 | ['PA(15:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))', 'PA(17:1(9Z)/20:5(5Z,8Z,11Z,14Z,17Z))', 'PA(17:2(9Z,12Z)/20:4(5Z,8Z,11Z,14Z))', 'PA(20:4(5Z,8Z,11Z,14Z)/17:2(9Z,12Z))', 'PA(20:5(5Z,8Z,11Z,14Z,17Z)/17:1(9Z))', 'PA(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/15:0)'] |
| 769.5565 | C46H80O6 | [M+(41K)]+ | 728.5955 | −0.35 | ['TG(12:0/13:0/18:4(6Z,9Z,12Z,15Z))[iso6]'] |
| 774.5042 | C39H78NO9P | [M+K]+ | 735.5414 | −0.54 | ['PS(O-16:0/17:0)', 'PS(O-18:0/15:0)', 'PS(O-20:0/13:0)'] |
| 807.5723 | C49H82O6 | [M+(41K)]+ | 766.6111 | −0.17 | ['TG(12:0/12:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))[iso3]', 'TG(12:0/14:1(9Z)/20:5(5Z,8Z,11Z,14Z,17Z))[iso6]', 'TG(14:1(9Z)/14:1(9Z)/18:4(6Z,9Z,12Z,15Z))[iso3]'] |
| 817.644 | C43H87N2O6P | [M + Hac-H]- | 758.6302 | −0.1 | ['SM(d16:1/22:0)', 'SM(d18:1/20:0)'] |
| 821.5529 | C43H81O12P | [M+H]+ | 820.5466 | −1.16 | ['PI(O-16:0/18:2(9Z,12Z))', 'PI(P-16:0/18:1(9Z))', 'PI(P-18:0/16:1(9Z))', 'PI(P-20:0/14:1(9Z))'] |
| 845.6628 | C48H93O9P | [M+H]+ | 844.6557 | −0.24 | ['PG(O-20:0/22:2(13Z,16Z))', 'PG(P-20:0/22:1(11Z))'] |
| 847.6793 | C48H95O9P | [M+H]+ | 846.6714 | 0.81 | ['PG(O-20:0/22:1(11Z))', 'PG(P-20:0/22:0)'] |
| 879.6719 | C49H99N2O6P | [M + K-2H]- | 842.7241 | −0.91 | ['SM(d18:0/26:1(17Z))', 'SM(d18:1/26:0)', 'SM(d20:0/24:1)'] |
| 948.6519 | C48H96NO13P | [M+Na]+ | 925.6619 | 0.76 | ['PI-Cer(t18:0/24:0(2OH))', 'PI-Cer(t20:0/22:0(2OH))'] |

**Table A.4**
PUTMEDID annotation of the non-polar peaks detected in human plasma via LC-MS and presented in Fig. A2 (b).

| *m/z* | rt | Adduct | Mass error (ppm) | Metabolite name |
|---|---|---|---|---|
| 402.3562 | 459.478 | NH3 | 3.84 | MG[20:1]; Tetrahydropersin; Tricosanedioic acid;; |
| 443.2041 | 495.6556 | H | 0.12 | PG[12:0];;Armillane;;"11,12-Dimethylrosmanol"; "11beta,20-Dihydroxy-3-oxopregn-4-en-21-oic acid"; 17-oxo-Resolvin D1; 6-methylprednisolone; 8-oxo-Resolvin D1; jasmolin II; Macrophorin B;; |
| 460.2805 | 509.4291 | Na | 1.42 | PE(P-16:0e/0:0);; |
| 511.2932 | 539.3329 | NaCl | 1.23 | PA(O-20:0/0:0);;" 1alpha,25-dihydroxy-26,27-dimethyl-17,20,22,22,23,23-hexadehydro-24a-homocholecalciferol"; " 1alpha,25-dihydroxy-26,27-dimethyl-20,21,22,22,23,23-hexadehydro-24a-homocholecalciferol"; " 24bE)-1alpha,25-dihydroxy-22,23,24,24a,24b,24c-hexadehydro-24a,24b,24c-trihomocholecalciferol"; "3-Oxo-12,18-ursadien-28-oic acid"; Tyromycic acid; VD 2728; VD 2736;;27-Deoxy-5alpha-cyprinol; 27-Deoxy-5b-cyprinol; "5a-Cholestane-3a,7a,12a,25-tetrol"; "5b-Cholestane-3a,7a,12a,23-Tetrol"; "5b-Cholestane-3a,7a,12a,25-tetrol"; "5beta-cholestane-3alpha,7alpha,12alpha,26-tetrol"; "Cholestane-3,7,12,25-tetrol"; Myxinol;; 1alpha-hydroxy-3-deoxycholecalciferol; 3-epicholecalciferol; 1-(5alpha)-cholestenone; 22-dehydrocholesterol; "24,26-cyclocholesterol"; 3-Deoxy-25-hydroxyvitamin D3; 3-epi-vitamin D3; 3-epicholecalciferol; 3-ketocholesterol; "4,6-cholestadienol"; "5, 20(22)-cholestadienol"; "5,6-trans-Vitamin D3"; "5a-Cholesta-7,24-dien-3b-ol"; 5alpha-Cholest-7-en-3-one; "5alpha-cholesta-7,24-dien-3beta-ol"; 5Alpha-cholesta-8-en-3-one; 7-Dehydrocholesterol; 8-Dehydrocholesterol; calciol; Cholestenone; Cystosterol; Dehydrocholesterol; Desmosterol; isocholecalciferol; isotachysterol3; Lumisterol 3; Occelasterol; Previtamin D3; tacalciol; Tachysterol 3; toxisterol3 D1; toxisterol3 E1; Vitamin D3; Zymosterol intermediate 2;; |
| 582.2923 | 490.9632 | NaCl | 1.77 | PE[20:0];;Ecalcidene;; |
| 589.3464 | 560.9315 | Na | 2.11 | PG[22:1];;"11alpha-Hemiglutaryloxy-1,25-dihydroxyvitamin D3"; Ganoderic acid Mi; Ganoderic acid Mj;;Didodecyl thiobispropanoate;;" 1alpha,22,25-trihydroxy-26,27-dimethyl-23,23,24,24-tetradehydro-24a,24b,24c-trihomocholecalciferol"; " 1alpha,22,25-trihydroxy-26,27-dimethyl-23,24-tetradehydro-24a,24b,24c-trihomo-20-epicholecalciferol"; " 1alpha,25-dihydroxy-22-ethoxy-26,27-dimethyl-23,24-tetradehydro-24a-homo-20-epicholecalciferol"; Acetylursolic acid; beta-Boswellic acid acetate; Tsugaric acid A; Ursololactone;; |
| 616.3472 | 521.6131 | NH3 | 2.46 | PI[18:1];; |
| 630.3016 | 521.4316 | Na_HCOONa | 4.20 | PS[19:0];; |
| 635.4608 | 592.7129 | Na_Na | 2.14 | DG[34:3];; |
| 651.4147 | 558.5981 | NaCl | 3.08 | PA(O-16:0/13:0);;Ubiquinol-6;;DG[33:3];;Anhydrorhodovibrin;;hexatriacontahexaenoic acid; Linolenyl linolenate; Retinyl palmitate;; |
| 740.5157 | 594.2042 | Na_HCOONa | 2.63 | PC(O-14:0/O-14:0);; |
| 751.5664 | 602.4034 | K | 3.50 | DG[43:5];; |
| 817.6449 | 657.7228 | HCOOH | 1.13 | SM(d16:1/23:0); SM(d17:1/22:0); SM(d18:1/21:0); SM(d19:1/20:0); SM(d20:1/19:0);; |
| 845.6356 | 638.5628 | HCOOH | 3.96 | PS(O-18:0/22:2); PS(O-20:0/20:2); PS(P-18:0/22:1); PS(P-20:0/20:1);;SM(d18:0/22:1(OH));; |
| 854.5483 | 531.7281 | Na_Na | 1.25 | PI-Cer(d18:0/18:0); PI-Cer(d20:0/16:0);; |
| 893.754 | 804.3246 | Na_Na | 0.49 | TG[53:3];;Glycerol triheptadecanoate;; |
| 900.6752 | 700.9033 | Na_Na | 4.45 | PC(24:0/P-18:1); PC(24:1/P-18:0); PC(o-18:2/24:0); PC(O-20:0/22:2); PC(P-18:0/24:1); PC(P-18:1/24:0); PC(P-20:0/22:1);; |
| 929.7527 | 745.4404 | Na_Na | 1.95 | TG[57:6];;TG[54:3]; TG[55:3]; Tripetroselinin;; |

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**Table A.5**
PUTMEDID annotation of the non-polar peaks detected in human urine via LC-MS and presented in Fig. A2 (c).

| *m/z* | rt | Adduct | Mass error (ppm) | Metabolite name |
|---|---|---|---|---|
| 453.3089 | 530.0064 | NH3 | 2.25 | PE(P-16:0e/0:0);; |
| 465.3921 | 574.9083 | HCOONa | 1.43 | MG[24:0];;N-arachidonoyl-dopamine-d8;;18-methyl-hexacosanoic acid; 18-methyl-pentacosanoic acid; "18,24-Dimethylhexacosanoic acid"; 23-methyl-pentacosanoic acid; 4-Methyl-3-heptyl stearate; 6-Hydroxy-8-hexacosanone; Hexacosanoic acid; Isocerotic acid; Lauryl myristate; Mycocerosic acid (C26); Mycosanoic acid (C26); Myristyl laurate;; |
| 511.2928 | 539.6198 | NaCl | 0.43 | PA(O-20:0/0:0);;" 1alpha,25-dihydroxy-26,27-dimethyl-17,20,22,22,23,23-hexadehydro-24a-homocholecalciferol"; " 1alpha,25-dihydroxy-26,27-dimethyl-20,21,22,22,23,23-hexadehydro-24a-homocholecalciferol"; " 24bE)-1alpha,25-dihydroxy-22,23,24,24a,24b,24c-hexadehydro-24a,24b,24c-trihomocholecalciferol"; "3-Oxo-12,18-ursadien-28-oic acid"; Tyromycic acid; VD 2728; VD 2736;;27-Deoxy-5alpha-cyprinol; 27-Deoxy-5b-cyprinol; "5a-Cholestane-3a,7a,12a,25-tetrol"; "5b-Cholestane-3a,7a,12a,23-Tetrol"; "5b-Cholestane-3a,7a,12a,25-tetrol"; "5beta-cholestane-3alpha,7alpha,12alpha,26-tetrol"; "Cholestane-3,7,12,25-tetrol"; Myxinol;; 1alpha-hydroxy-3-deoxycholecalciferol; 3-epicholecalciferol; 1-(5alpha)-cholestenone; 22-dehydrocholesterol; "24,26-cyclocholesterol"; 3-Deoxy-25-hydroxyvitamin D3; 3-epi-vitamin D3; 3-epicholecalciferol; 3-ketocholesterol; "4,6-cholestadienol"; "5, 20(22)-cholestadienol"; "5,6-trans-Vitamin D3"; "5a-Cholesta-7,24-dien-3b-ol"; 5alpha-Cholest-7-en-3-one; "5alpha-cholesta-7,24-dien-3beta-ol"; 5Alpha-cholesta-8-en-3-one; 7-Dehydrocholesterol; 8-Dehydrocholesterol; calciol; Cholestenone; Cystosterol; Dehydrocholesterol; Desmosterol; isocholecalciferol; isotachysterol3; Lumisterol 3; Occelasterol; Previtamin D3; tacalciol; Tachysterol 3; toxisterol3 D1; toxisterol3 E1; Vitamin D3; Zymosterol intermediate 2;; |
| 561.3157 | 530.5856 | HCOONa | 1.10 | PG[20:1];;Phytolaccinic acid; Pokeberrygenin;;PA[22:1];;" 1alpha-hydroxy-18-(4-hydroxy-4-ethyl-2-hexynyloxy)-23,24,25,26,27-pentanorcholecalciferol"; " 1alpha,22,25-trihydroxy-26,27-dimethyl-23,23,24,24-tetradehydro-24a-homocholecalciferol"; " 1alpha,22,25-trihydroxy-26,27-dimethyl-23,24-tetradehydro-24a-homo-20-epicholecalciferol"; " 1alpha,25-dihydroxy-22-methoxy-26,27-dimethyl-23,23,24,24-tetradehydrocholecalciferol"; " 1alpha,25-dihydroxy-22-methoxy-26,27-dimethyl-23,24-tetradehydro-20-epicholecalciferol"; "(3alpha,3-Hydroxy-21-oxoeupha-8,24-dien-26-oic acid"; 11-Keto-beta-boswellic acid; 16-Hydroxy-3-oxo-12-oleanen-28-oic acid; 2-Hydroxy-3-oxo-12-oleanen-28-oic acid; 23-Hydroxy-3-oxocycloart-24-en-26-oic acid; 28-Hydroxymangiferonic acid; 3alpha-hydroxyglycyrrhetinic acid; 6beta-Hydroxy-3-oxo-12-oleanen-28-oic acid; beta-Glycyrrhetinic acid; Colubrinic acid; Glycyrrhetinic acid; Glycyrrhetinic acid; gypsogenin; Koetjapic acid; Lansic acid; Liquiritic acid; Murrayenol; Nebrosteroid L; Pomonic acid; Rubinic acid;; |
| 587.4056 | 604.7231 | K | 2.82 | DG[31:3];; |
| 612.3629 | 500.6091 | Na_HCOONa | 2.78 | PC(O-18:1/O-1:0);; |
| 637.3958 | 549.3790 | NaCl | 2.00 | PA(O-16:0/12:0);; |
| 678.3885 | 532.1264 | NaCl | 1.90 | PE[27:1];; |
| 702.4695 | 541.0185 | HCOONa | 0.58 | PS(P-16:0/15:1);;PE(O-16:0/13:0);; |
| 750.5169 | 600.0387 | NaCl | 0.73 | PC(o-14:0/16:0); PC(O-16:0/14:0); PC(O-18:0/12:0); PE(O-16:0/17:0); PE(O-18:0/15:0); PE(O-20:0/13:0);; |
| 756.4676 | 529.2469 | NH3 | 2.47 | PI[27:1];;PE(18:3/P-16:0); PE(O-16:0/18:4); PE(P-16:0/18:3);; |
| 757.6071 | 729.1700 | Na_HCOONa | 1.28 | DG[43:2];;Campesteryl ester[20:3];;Campesteryl ester[18:0]; Campesteryl stearate; CE[19:0];; |
| 845.6369 | 638.8819 | HCOOH | 2.43 | PS(O-18:0/22:2); PS(O-20:0/20:2); PS(P-18:0/22:1); PS(P-20:0/20:1);;TG[48:2];;SM(d18:0/22:1(OH));; |
| 859.6027 | 549.9083 | NH3 | 0.92 | PI-Cer(t18:0/18:0(2OH)); PI-Cer(t20:0/16:0(2OH));;SM(d18:0/22:1(OH));;PG(O-16:0/22:1); PG(O-18:0/20:1); PG(O-20:0/18:1); PG(P-16:0/22:0); PG(P-18:0/20:0); PG(P-20:0/18:0);;TG[48:8];;TG[46:5];; |
| 869.6239 | 633.0871 | HCOONa | 0.38 | PG[43:4];;PG[41:1];;Glc-Campesterol[18:2];;SM(d18:2/24:1);;PA[43:1];;TG[47:4];; |
| 907.7161 | 632.9944 | K | 1.03 | TG[53:4]; TG[55:4];; |
| 965.7323 | 805.5465 | NaCl | 1.34 | TG[57:6];; |

**Table A.6**
MI-Pack annotation of the polar peaks detected in *D. magna* via DIMS and presented in Fig. A3 (a).

| *m/z* | Empirical formula | Ion form | Theoretical mass (Da) | Mass error (ppm) | Metabolite name |
|---|---|---|---|---|---|
| 84.0421 | C2H7NO | [M+Na]+ | 61.0528 | 0.89 | ['Ethanolamine'] |
| 90.0549 | C3H7NO2 | [M+H]+ | 89.0477 | −0.73 | ['D-Alanine', 'L-Alanine', 'Sarcosine', 'beta-Alanine'] |
| 98.0212 | C2H5NO2 | [M+Na]+ | 75.0320 | −0.51 | ['Glycine'] |
| 114.0560 | C5H9NO2 | [M − H]- | 115.0633 | −0.37 | ['D-Proline', 'L-Proline'] |
| 116.0717 | C5H11NO2 | [M − H]- | 117.0790 | −0.11 | ['Betaine', 'L-Valine'] |
| 120.0125 | C3H7NO2S | [M − H]- | 121.0198 | 0.04 | ['L-Cysteine'] |
| 120.0654 | C4H9NO3 | [M+H]+ | 119.0582 | −0.92 | ['L-Allothreonine', 'L-Homoserine', 'L-Threonine'] |
| 124.0073 | C2H7NO3S | [M − H]- | 125.0147 | −0.56 | ['Taurine'] |
| 128.0317 | C3H7NO3 | [M+Na]+ | 105.0426 | −1.06 | ['D-Serine', 'L-Serine'] |
| 131.0461 | C4H8N2O3 | [M − H]- | 132.0535 | −0.66 | ['3-Ureidopropionate', 'L-Asparagine', 'N-Carbamoylsarcosine'] |
| 132.0302 | C4H7NO4 | [M − H]- | 133.0375 | −0.55 | ['L-Aspartate'] |
| 132.1018 | C6H13NO2 | [M+H]+ | 131.0946 | −1.1 | ['6-Aminohexanoate', 'L-Isoleucine', 'L-Leucine'] |
| 147.0765 | C5H10N2O3 | [M+H]+ | 146.0691 | 0.21 | ['(R)-3-Ureidoisobutyrate', 'D-Glutamine', 'L-Glutamine'] |
| 147.1128 | C6H14N2O2 | [M+H]+ | 146.1055 | −0.23 | ['L-Lysine'] |
| 150.0583 | C5H11NO2S | [M+H]+ | 149.0511 | 0.08 | ['L-Methionine'] |
| 154.0475 | C5H9NO3 | [M+Na]+ | 131.0582 | 0.16 | ['(S)-4-Amino-5-oxopentanoate', '5-Amino-2-oxopentanoic acid', '5-Aminolevulinate', 'Hydroxyproline', 'L-Glutamate 5-semialdehyde'] |
| 156.0768 | C6H9N3O2 | [M+H]+ | 155.0695 | 0.43 | ['L-Histidine'] |
| 163.1077 | C6H14N2O3 | [M+H]+ | 162.1004 | 0 | ['L-Hydroxylysine'] |
| 166.0863 | C9H11NO2 | [M+H]+ | 165.0790 | 0.21 | ['L-Phenylalanine'] |
| 172.0018 | C4H9NO2S | [M+(37Cl)]- | 135.0354 | −0.54 | ['L-Homocysteine'] |
| 175.1190 | C6H14N4O2 | [M+H]+ | 174.1117 | 0.1 | ['D-Arginine', 'L-Arginine'] |
| 182.0812 | C9H11NO3 | [M+H]+ | 181.0739 | 0.11 | ['L-Tyrosine'] |
| 205.0972 | C11H12N2O2 | [M+H]+ | 204.0899 | 0.03 | ['L-Tryptophan'] |
| 227.1141 | C9H14N4O3 | [M+H]+ | 226.106,591 | 0.98 | ['Carnosine'] |

Please cite this article in press as: J. Sostare, et al., Comparison of modified Matyash method to conventional solvent systems for polar metabolite and lipid extractions, Analytica Chimica Acta (2018), https://doi.org/10.1016/j.aca.2018.03.019

**Table A.7**
PUTMEDID annotation of the polar peaks detected in human plasma via LC-MS and presented in Fig. A3 (b).

| *m/z* | rt | Adduct | Mass error (ppm) | Metabolite name |
|---|---|---|---|---|
| 132.1020 | 404.2944 | NH3 | 0.90 | Beta-Leucine; D-Leucine; L-Alloisoleucine; L-Norleucine;;2-Methyl-2-pentenoic acid; 2-Methyl-3-pentenoic acid; 2-Methyl-4-pentenoic acid; "2,3-Hexanedione"; 3-isopropyl acrylic acid; "3,4-Hexanedione"; 4-hydroxyhexenal; 4-Methyl-2-pentenoic acid; "4-Methyl-2,3-pentanedione"; Adipaldehyde; Allyl propionate; Delta-Hexanolactone; delta-hexenoic acid; Ethyl crotonate; Gamma-Caprolactone; gamma-hexenoic acid; Hydrosorbic acid; Methyl 4-pentenoate; Prenyl formate; Pyroterebic acid; trans-Hex-2-enoic acid; trimethyl acrylic acid;; |
| 137.0927 | 73.7697 | NH3 | 4.66 | L-Homoserine; L-Allothreonine;; |
| 147.0766 | 602.4155 | [M+H]+ | 0.89 | L-Glutamine; Alanylglycine; D-Glutamine; Ureidoisobutyric acid;; |
| 147.1130 | 714.1273 | [M+H]+ | 1.53 | L-Lysine; 3,5-Diaminohexanoate; "3,6-Diaminohexanoate"; D-Lysine;; |
| 148.0605 | 625.9020 | [M+H]+ | 0.23 | L-Glutamic acid; L-4-Hydroxyglutamate semialdehyde; N-Acetylserine; O-Acetylserine;; |
| 150.0140 | 608.9164 | Na_HCOONa | 1.93 | L-Serine; D-Serine;;Acetaldehyde oxime; Acetamide; N-Methylformamide;; |
| 159.0766 | 623.7098 | [M+H]+ | 0.90 | Citrulline; Argininic acid; "1-(Hydroxymethyl)-5,5-dimethyl-2,4-imidazolidinedione";; |
| 161.9931 | 609.8744 | Na | 4.40 | O-Phosphoethanolamine;; |
| 173.1037 | 698.3409 | H | 4.16 | L-Arginine; D-Arginine;; |
| 189.1348 | 684.9616 | [M+H]+ | 1.03 | Homo-L-arginine; L-Targinine;; |
| 199.9544 | 827.3276 | KCl | 0.65 | Taurine;; |
| 205.0972 | 379.3728 | [M+H]+ | 0.33 | (±)-Tryptophan; 3-Hydroxymethylantipyrine; D-Tryptophan;; |
| 207.9983 | 627.1723 | [M+Na]+ | 0.59 | Phosphoserine; DL-O-Phosphoserine; |
| 240.1019 | 317.3329 | NH3 | 2.44 | L-Cystathionine; Allocystathionine; Cysteinyl-Threonine; Threoninyl-Cysteine;; |
| 241.0315 | 762.1263 | [M+H]+ | 1.22 | L-Cystine;; |
| 244.0242 | 792.6005 | KCl | 3.05 | S-(3-oxo-3-carboxy-n-propyl)cysteine;;1-Methylhistidine; 3-Methylhistidine;;Nor-psi-tropine;; |
| 313.0523 | 238.1227 | Na_Na | 2.21 | L-Homocystine; Aspartyl-Histidine; Histidinyl-Aspartate;;Temurin;;"7-Isopropyl-1,4-dimethylazulene";;DL-Homocystine;; |

**Table A.8**
PUTMEDID annotation of the polar peaks detected in human urine via LC-MS and presented in Fig. A3 (c).

| *m/z* | rt | Adduct | Mass error (ppm) | Metabolite name |
|---|---|---|---|---|
| 104.0707 | 510.2082 | NH3 | 0.99 | b-aminoisobutyric acid; 2-Aminoisobutyric acid; 3-Aminobutanoic acid; 3-Aminoisobutanoic acid; Butyl nitrite; D-Alpha-aminobutyric acid; Dimethylglycine; Gamma-Aminobutyric acid; L-Alpha-aminobutyric acid; N-Ethylglycine;;But-2-enoic acid; Diacetyl; Ethenyl acetate; Gamma-Butyrolactone; Isocrotonic acid; Oxolan-3-one; Vinyl acetic acid;; |
| 106.0501 | 632.0966 | NH3 | 1.96 | L-Serine; D-Serine;;Malonic semialdehyde; Pyruvic acid;; |
| 122.0268 | 325.2378 | H | 1.78 | L-Cysteine; D-Cysteine;; |
| 126.0217 | 475.9900 | H | 2.41 | Taurine;; |
| 132.1015 | 585.9744 | NH3 | 3.01 | Beta-Leucine; D-Leucine; L-Alloisoleucine; L-Norleucine;;2-Methyl-2-pentenoic acid; 2-Methyl-3-pentenoic acid; 2-Methyl-4-pentenoic acid; "2,3-Hexanedione"; 3-isopropyl acrylic acid; "3,4-Hexanedione"; 4-hydroxyhexenal; 4-Methyl-2-pentenoic acid; "4-Methyl-2,3-pentanedione"; Adipaldehyde; Allyl propionate; Delta-Hexanolactone; delta-hexenoic acid; Ethyl crotonate; Gamma-Caprolactone; gamma-hexenoic acid; Hydrosorbic acid; Methyl 4-pentenoate; Prenyl formate; Pyroterebic acid; trans-Hex-2-enoic acid; trimethyl acrylic acid;; |
| 133.0607 | 606.9302 | H | 0.62 | L-Asparagine; Glycyl-glycine; D-Asparagine; N-Carbamoylsarcosine; Ureidopropionic acid;;1-Pentanol; 2-Methyl-1-butanol; Isopentanol;; |
| 134.0184 | 552.1245 | Na_Na | 3.45 | L-Alanine; Beta-Alanine; D-Alanine; Ethyl carbamate; Sarcosine;; |
| 147.0759 | 597.3338 | H | 3.52 | L-Glutamine; Alanylglycine; D-Glutamine; Ureidoisobutyric acid;; |
| 147.1123 | 708.9655 | NH3 | 3.44 | L-Lysine; 3,5-Diaminohexanoate; "3,6-Diaminohexanoate"; D-Lysine;;2-Pyrrolidineacetic acid; D-Pipecolic acid; L-Pipecolic acid; L-trans-4-Methyl-2-pyrrolidinecarboxylic acid; N4-Acetylaminobutanal; Pipecolic acid;; |
| 162.0755 | 614.0318 | H | 3.57 | Aminoadipic acid; Acetylhomoserine; 2-Methylglutaconic acid; 3-Hexenedioic acid; "3-Hydroxyadipic acid 3,6-lactone"; 3-Methylglutaconic acid; Dimethyl fumarate; Ethyl hydrogen fumarate; Maleic acid homopolymer; trans-2-Hexenedioic acid;; |
| 164.0286 | 568.8984 | Na_HCOONa | 4.73 | L-Homoserine; L-Allothreonine; 3-Aminopropionaldehyde; Aminoacetone;; |
| 173.1042 | 690.3098 | H | 1.17 | L-Arginine; D-Arginine;; |
| 176.1030 | 551.2034 | H | 0.04 | Citrulline; Argininic acid;; |
| 178.0082 | 450.0013 | Na_Na | 2.62 | L-Aspartic acid; Iminodiacetic acid; D-Aspartic acid;; |
| 180.0659 | 453.1635 | HCOOH | 4.20 | L-Tyrosine; 4-Hydroxy-4-(3-pyridyl)-butanoic acid; Beta-Tyrosine; L-Threo-3-Phenylserine; o-Tyrosine;;2-Aminoacetophenone; 2-Phenylacetamide; 2'-Aminoacetophenone; N-Acetylarylamine;; |
| 201.0851 | 442.0920 | HCOONa | 2.55 | L-Ornithine; D-Ornithine monochlorohydrate/ornithine; Ornithine;; |
| 223.0740 | 744.1265 | Na | 4.88 | L-Cystathionine; Allocystathionine; Cysteinyl-Threonine; Threoninyl-Cysteine;;3-(1-Butenyl)-1H-2-benzopyran-1-one; Safynol;; |
| 241.0310 | 753.4439 | H | 0.77 | L-Cystine;; |
| 273.0837 | 260.4907 | HCOONa | 3.40 | (±)-Tryptophan; 3-Hydroxymethylantipyrine; D-Tryptophan;;Alanyl-Valine; Glycyl-Isoleucine; Glycyl-L-leucine; Isoleucyl-Glycine; Leucyl-Glycine; N-Alpha-acetyllysine; N6-Acetyl-L-lysine; Valyl-Alanine;;"1,3-Octanediol"; "octane-1,2-diol";; |
| 313.0530 | 358.1893 | Na_Na | 0.17 | L-Homocystine; Aspartyl-Histidine; Histidinyl-Aspartate;;Temurin;;DL-Homocystine;; |

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Please cite this article in press as: J. Sostare, et al., Comparison of modified Matyash method to conventional solvent systems for polar metabolite and lipid extractions, Analytica Chimica Acta (2018), https://doi.org/10.1016/j.aca.2018.03.019